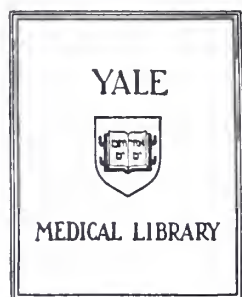


THE EFFECT OF PROTONS ON PHOTOTRANSDUCTION IN
THE ROD OUTER SEGMENT OF BUFO MARINAS

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The Effect of Protons on Phototransduction in
the Rod Outer Segment of Bufo Marinas

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TABLE OF CONTENTS

Abstract.....	iv
Introduction.....	1
General Problem Description.....	1
Microscopic Anatomy.....	2
Visual Pigments and the Biochemisry of Visual Excitation.....	4
Electrophysiology of Vertebrate Rod Outer Segments.....	7
The Evaluation of Internal Transmitters.....	12
Materials and Methods.....	20
Results.....	23
The Effects of Proton Injection on Resting Membrane Potential.....	23
The Effects of Proton Injection on the Light Response.....	24
The Effects of Proton Injection on During Recovery.....	26
Discussion.....	28
Figures.....	40
Appendix.....	64
References.....	65

ABSTRACT

A dark adapted vertebrate rod cell, when exposed to a light flash, transiently hyperpolarizes (1). Light energy is captured by the photopigment, rhodopsin which is located in the rod disc membranes. The discs are physically and electrically isolated from the plasma membrane. Ca (53) and cGMP (46,65) have been proposed as the internal messengers by which light capture by rhodopsin is communicated to the plasma membrane.

It has been suggested that protons produced from the hydrolysis of cGMP may induce the release of Ca from intracellular stores (4). Though this theory is no longer valid since cGMP has been determined to function as the internal transmitter (2,3), that protons may cause local changes in pH and have a role in phototransduction is plausible. It is the purpose of this thesis to investigate the effect of protons on vertebrate phototransduction.

Previous attempts to assess the function of protons in the rod outer segment have yielded inconsistent results. Pinto and Ostroy (83) find protons depolarize, MacLeish et al. (84) find no effect, and Mueller and Pugh (4) and Liebman et

al. (85) find protons decrease the dark current. My results are consistent with the latter findings. In addition, I report here the new finding that protons hyperpolarize with no measurable latency. This new result leads me to suggest that protons act directly on the plasma membrane rather than through Ca or the cyclic nucleotide cascade. Whether protons have a regulatory role in the transduction process has yet to be tested.

Others have argued that the buffering power of the rod cell prevents significant changes in pH (85). Determination of whether the results reported here are artifactual or physiologic will depend on pH measurements under more physiologic conditions and evaluation of the pH dependence of the cationic channels.

I. INTRODUCTION

A. General Problem Description

The retina of the human eye contains approximately 125 million highly specialized cells for the detection of light. These cells, working in cooperation with others in the retina and brain, are so intricate and versatile that they facilitate a three dimensional reconstruction of the surrounding world in the mind. The photoreceptor rod cell is so exquisitely sensitive that when properly dark-adapted it is able to respond to a single photon. Rhodopsin, a transmembrane photopigment located in the rod discs, is able to absorb a photon which causes isomerization of its chromophore. A light pulse causes a transient hyperpolarization along the plasma membrane of the rod cell (1) and this excitation is eventually communicated to the higher neurons of the visual system. The molecular mechanism of sensory transduction in rod cells, or phototransduction, the process by which light energy is converted to electrical signals, is incompletely understood.

Recent data from patch clamp experiments (2,3) have shown cyclic guanosine 3',5'-monophosphate (cGMP) to be the final internal messenger in the phototransduction process. Pre-

viously, calcium has been considered to play a similar role and numerous studies support the notion that Ca is, in some manner, involved in the phototransduction process. One hypothesis proposes that the coupling of photon capture and its translation into an electrical signal is dependent on protons generated from the cleavage of cGMP by phosphodiesterase, PDE. The protons would then induce the release of Ca from intracellular storage sites (4). It is the purpose of my experiments to more closely examine the nature of the effects of protons and their role in sensory transduction in the vertebrate rod cell.

B. Microscopic Anatomy

The retina is located on the inner posterior surface of the eye; the photoreceptor cells are organized in a single layer in the back of the retina. Rod cells are long, slender cells composed of several specialized compartments (Fig. 1). At one extreme, away from the lens, is located the outer segment, a modified cilium, which is highly specialized for the capture of light energy. These rod outer segments (ROS) contain an elaborate system of 1000-2000 vertically stacked membranous discs on which is located the visual photopigment, rhodopsin. This particular arrangement maximizes the probability for photon capture by rod cells and

constitutes a basic element in the sensitivity to visual images. Photons traverse the waveguide ROS by total internal reflection; thus, light that escapes one disc may be captured by another within the same rod cell. All but a few basal discs are physically separated from the plasma membrane; most are true free-floating discs within the cell cytoplasm (Fig. 2). The discs are formed at the base of the ROS as a series of invaginations of the plasma membrane. The invaginations pinch off and separate completely from the plasma membrane (5) as new discs are formed.

The discs of the ROS undergo constant renewal; in autoradiographic studies (6), Droz showed that the membranous discs are constantly synthesized as the discs migrate from the base to the tips. Within minutes, radioactively labeled amino acids are seen in the inner segment; over a period of four days, the synthesized proteins migrate from the base to the tips of ROS. Conversely, the tips of the ROS are removed by the phagocytic activity of the contiguous pigment epithelial cells (7); phagocytosis is stimulated by the first exposure to light each morning (8).

The ROS is connected to the inner segment through a narrow ciliary stalk. This bridge contains a modified cilium of nine pairs of microtubules but lacking the central pair of

microtubules characteristic of motile cilia. The inner segment contains the metabolic and synthetic machinery, the nucleus, numerous mitochondria, ribosomes, and Golgi complex, for the maintenance of the highly metabolically active cell. The inner segment is contiguous with the most basal portion of the rod cell which forms the synaptic terminal. This segment of the rod cell relays the electrical signal to the adjoining neurons, the horizontal and bipolar cells which comprise the first steps of the neural processing and integration of visual stimuli.

C. Visual Pigments and the Biochemistry of Visual Excitation

The visual pigments consist of the chromophore, retinal, bound to the apoprotein, opsin, by a Schiff-base linkage. In vertebrate rod cells, the pigment is rhodopsin in which 11-cis retinal functions as the chromophore. Rhodopsin is located in the discs of the ROS and its structural relation to the discs has been extensively studied. There are approximately one billion rhodopsin molecules in each rod cell. It exists as a single polypeptide of molecular weight 38 kilodaltons (9). X-ray (10) and neutron diffraction (11), freeze fracture electron microscopy (12), nuclear magnetic resonance spectroscopy (13), and radioactive labeling (9), have demonstrated that rhodopsin is an

integral protein that traverses the membrane seven times (14). Opsin has recently been sequenced (15) and its structure has been characterized.

By inspection of the retinal molecule, one might suspect the most thermodynamically stable form is the all-trans retinal since this isomer minimizes steric hindrance of the methyl groups. However, in darkness, 11-cis retinal is found to be relatively stable (16) and is the predominant isomer despite the overlap of the C-13 methyl group and C-10 hydrogen. But, when retinas are bleached by light, the all-trans retinal becomes the predominant form. This isomerization cycle is the first activation step of excitation.

George Wald in a series of cooling and warming experiments was able to identify many of the intermediates (16). In darkness, rhodopsin exists in the 11-cis retinal form; exposed to light, it is transformed to the all-trans isomer through a series of unstable intermediates, referred to as the bleaching sequence. The isomerization of a chromophore is the only light dependent stage in visual excitation. All the remaining chemical and physiologic reactions that occur in response to light stimuli are a consequence of this one isomerization (17). The first intermediate is bathorhodopsin (18) though hypsorhodopsin may be a precursor (19).

Once bathorhodopsin is formed, opsin undergoes conformational changes to rapidly form intermediates lumirhodopsin, metarhodopsin I, metarhodopsin II, and metarhodopsin III at neutral pH (20). Finally, the Schiff base linkage undergoes hydrolysis to yield all-trans retinal and opsin (21).

The all-trans retinal is reduced to all-trans retinol in the ROS and transported to the pigment epithelium by a transport protein. There it is reisolomerized to 11-cis retinol (22). 11-cis retinol in the pigment epithelium can have two fates. It can be esterified to form retinyl esters which constitute the major retinoid in the pigment epithelium or it may be reisolomerized to the 11-cis retinol and transported back to the ROS where it may be oxidized to the retinal form. Opsin has a very high affinity for the 11-cis retinal (23), thus, although the equilibrium for retinol-retinal greatly favors retinol, effectively all the 11-cis retinal is trapped by the opsin, hence, the reaction is pulled toward the retinal form. A retinal binding protein also facilitates rhodopsin formation (24). Thus, on a bright day, much of the rhodopsin in the rods is broken down and the retinal extruded from the ROS. In dimmer light or darkness, there is net rhodopsin synthesis. The fluctuations in rhodopsin concentration in ROS are the biochemical basis of the phenomena of photochemical adaptation.

D. Electrophysiology of the Vertebrate Rod Outer Segment

In 1964, Tomita was the first to succeed at intracellular recording of single photoreceptor cells (1). As expected, when the microelectrode was introduced into the cell, a negative potential shift was observed. Unexpectedly, however, the potential was much less negative, in the (-30)-(-40) mV range and not (-60)-(-70) mV as in most other nerve cells. When exposed to a flash of light, the rod cell plasma membrane was observed to hyperpolarize to near -70mV. This is in contrast to the reactive depolarization in most other neural tissue. Subsequently it has been shown that the relatively small resting potential is due to a relatively high plasma membrane permeability to Na (25,26). Therefore, since the plasma membrane was unusually permeable to Na in the resting state, the resting potential of the rod cell is between the equilibrium potential of Na, estimated to be near 0 and 10 mV, and the equilibrium potential of K, approximately -90 mV. Further investigation has shown the hyperpolarizations to be associated with an increase in membrane resistance (27). The amplitude of the light response was linearly proportional to the logarithm of the extracellular Na concentration (28). These data suggest the receptor potential was the result of a decrease in Na permeability of the outer membrane. Others have confirmed

this observation and conclusion (25,26); in darkness, approximately one billion Na pass the outer membrane per second; in light, this flux is reduced by ten million Na per second per photolyzed rhodopsin in the frog (26).

The Na channel of the ROS facilitates the passive diffusion of Na; this dark current is unaffected in the short term by metabolic poisons (29) such as ouabain. As Na enters the cell, K leaks out of the cell down its electrochemical gradient. On the inner segment of the rod cell are located Na-K exchange pumps. This exchanger pumps Na out of the cell and pumps K back into the cell. Thus, the Na current runs extracellularly from inner to outer segment and intracellularly from the outer to inner segment. Along with Na, Ca also enters the ROS by passive diffusion through the Na channels. A Na-Ca exchange mechanism present on the ROS plasma membrane pumps Ca into the extracellular space in exchange for Na. Since extracellular Na concentration always exceeds the intracellular concentration, the function of the pump is dependent on the intracellular Ca concentration (30).

Interestingly, further study of the nature of the dark current has shown that the Na channel is, in fact, non-specific. Rapid exchange perfusion of ROS in physiologic Ca

concentrations has shown the ion selectivity of the channel to Li:Na:K:Rb:Cs to be as follows: 1.4:1.0:0.8:0.6:0.15 (31). After exposure to low Ca perfusion, the Na channel is selective for Ca vs. Na by nearly a factor of 9 (31). The Na channel facilitates Ca entry into the ROS as well as Na; the Ca contribution to the dark current has been estimated to be 10% (30). The Na channels would be more properly be thought of as light sensitive cationic channels.

The ionic basis for the receptor potential is relatively well characterized as is the behavior of rhodopsin to light. But the mechanism by which these two processes are coupled remained elusive until recently. Rhodopsin is located in the discs which are physically (5), electrically (32), and osmotically (33) isolated from the outer membrane and yet mediate the receptor potential on the plasma membrane. Clearly, to relate these two separate processes, an internal messenger must be postulated and on this point there is general agreement (34,35).

The search for the internal messenger has spurred work that has elucidated a more detailed understanding of the biochemistry of vision. The absorption of just one photon closes many Na channels. The absorption of one photon reduces this current by one pA (36) from its dark current of

20 pA, implying that a large amplification step or steps must be an integral part of the molecular mechanism responsible for transduction (37).

Light activated rhodopsin, probably metarhodopsin II (38), catalyzes the exchange of hundreds of guanine diphosphate, GDP, for guanine triphosphate, GTP, on transducin (Fig.3), a guanine nucleotide binding protein (39). Transducin, also known as G protein, is a ROS cytoplasmic protein that becomes bound to the inner discs when exposed to light (40). It is a protein complex composed of three polypeptide chains, T-alpha, T-beta, and T-gamma which are 39, 36, and 10 kilodaltons respectively (41). The binding of guanine nucleotide binding site is located on the alpha subunit of transducin (41). Photolyzed rhodopsin causes this exchange is by direct interaction with transducin (42). The lipids of the inner discs have a high degree of unsaturation (43) and, consequently, the membrane is very fluid (44). A single photon may catalyze the exchange of half of all the guanyl nucleotides bound to the discs (45). Fung and Stryer have isolated transducin (46) and, interestingly, found guanine triphosphatase, GTPase, activity (47) also located on the alpha subunit. This enzymatic activity plays a vital role in the completion of the light-dark cycle as discussed below.

A cyclic nucleotide phosphodiesterase, PDE, is also a ROS enzyme located on the discs (46). PDE is a holoenzyme composed of three subunits of molecular weight 88,84, and 13 kilodaltons (47). PDE is activated by GTP- α -transducin (41) which acts to relieve the inhibitory constraint (48) of the 13 kilodalton peptide (49).

Light induced PDE activation produces tremendous amplification; Yee and Liebman have demonstrated that one photolyzed rhodopsin results in the hydrolysis of 400,000 cGMP per second (41). PDE activity is terminated by hydrolysis of GTP to GDP by transducin GTPase (41) and perhaps by the phosphorylation of rhodopsin (50). Opsin kinase, a light activated ROS cytoplasmic enzyme, phosphorylates bleached rhodopsin. Phosphorylated rhodopsin is incapable of inducing GDP-GTP exchange on transducin (51), thus, photolyzed rhodopsin is inactivated. A light activated guanylate cyclase located in the inner segment (52) regenerates cGMP. The inactivation of PDE, phosphorylation of rhodopsin, and the activation of guanylate cyclase serve to attenuate the light response and reset the photolyzed rod cell to the dark state.

To summarize, the rod is depolarized by cGMP which increases the cationic permeability. Photolyzed rhodopsin induces the

exchange of GDP for GTP on hundreds of transducin molecules. The GTP-transducin, in turn, activates PDE which hydrolyzes thousands of cGMP to GMP. Thus, the absorption of a single photon results in the cleavage of hundreds of thousands of cGMP producing the light response by reducing the cGMP mediated Na permeability. The light response is inactivated by the GTPase activity of transducin which inactivates PDE, and probably by rhodopsin phosphorylation. cGMP is regenerated in the outer segment. The light sensitivity of the rod cell is controlled on three levels. First, light flashes decrease the ROS cGMP concentration. Second, opsin kinase phosphorylates rhodopsin rendering photolyzed rhodopsin incapable of activating transducin by catalyzing GDP-GTP exchange. Third, rhodopsin breaks down to yield opsin and all-trans retinal.

E. The Evaluation of Internal Transmitters

The light activated amplification cycle (Fig. 3) has been extensively studied (39). Yet there was no link to the plasma membrane; all the components of the cycle are located on the discs and the identity of the internal messenger remained unclear for some time. Research has concentrated on two candidates for consideration as internal messengers: cGMP and Ca. The criteria that any messenger must fulfill

to be considered in the phototransduction process has been reviewed (37). To summarize, transmitter must fulfill the following points:

1. must be present in the ROS
2. transmission is triggered by light activated rhodopsin
3. must account for the tremendous amplification of the phototransduction process
4. latency of reaction must be shorter than that of the receptor potential
5. must control the light dependent conductance
6. must control the kinetics of the light response
7. must control the latency of the light response

In 1971, Hagins and Yoshikami found Ca, topically applied to ROS, caused a decrease in Na permeability (53). They postulated that Ca is released by the in discs in response to light and blocks the Na channels. However, subsequent studies have proven that Ca does not fulfill the established criteria for internal messengers; the sum of evidence in favor of Ca as internal transmitter is wholly unconvincing and recent investigations have challenged many of the earlier findings or are inconsistent with the Ca hypothesis. Thus, the role of Ca in vertebrate photoreceptor cells remains to be established.

Early osmotic measurements found that Ca reduced Na permeability in plasma membrane (54). Ca was found in the ROS in sufficient concentration to function as a transmitter (55), free concentration of Ca was thought to be in the micromolar range (56). Increase in Ca concentration was reported to be a light induced phenomenon that preceeded the receptor potential (57). Ca efflux into the extracellular space was observed after light absorption. Szutz and Cone observed 10-1000 Ca released per photolyzed rhodopsin (60). EGTA, a Ca ion chelator-buffer, when introduced into the ROS attenuated the receptor potential (58). More recent data, however, have failed to confirm many of these observations and are inconsistent with the notion that Ca mediates the light response. Whether Ca was released in sufficient quantities to function as the final messenger was never firmly established (59). Some have observed that only 0.75 Ca are released for every photolyzed rhodopsin (59); it has been estimated that at least 100 transmitter molecules per photolyzed rhodopsin need to be released in order to block sufficient Na channels to result in the observed hyperpolarizations of the plasma membrane (61). Attempts to measure the light induced Ca release from discs have failed to show Ca release, fast or slow (62). Most troublesome, however, is the recent report that intracellular Ca de-

creases in response to light (63) and recovers only in the dark. Also, the Ca hypothesis implies that Ca is stored within the ROS presumably in an intracellular compartment. Though some data suggest that Ca is found in high concentration in the discs (55), light induced Ca flux across the disc membranes has not been documented (64). In short, Ca no longer seems tenable as internal messenger.

Cyclic nucleotides were proposed as the internal transmitter since their concentration was known to be regulated by light (65). PDE was initially thought to act on cAMP (65); however, it was later found that PDE hydrolyzes cGMP at a rate twenty three times that of cAMP (46). Originally, cGMP was thought to mediate changes in Na conductance by the mechanism of action of the other cyclic nucleotides by controlling phosphorylation of key proteins. Previous theories held that cGMP levels, in darkness, were high and that the Na channel was kept in the phosphorylated, open, state by cGMP through a protein kinase; in light, cGMP levels were thought to drop and the channel would then be dephosphorylated and closed.

However, Fesenko et al. (2) have found that the cGMP induced cationic conductance occurs in the absence of nucleoside triphosphates. This implies that the cGMP effect is not

mediated by protein phosphorylation; instead, it seems that cGMP modulates cationic permeability by direct interaction with plasma membrane proteins.

Early attempts to measure the dark ROS cGMP concentration estimated the total concentration, protein bound and free, to be in the 50 micromolar range (66). This was several times the concentration of cyclic nucleotides in other systems. It has been argued that PDE may be too slow to hydrolyze enough cGMP's in this large pool of cGMP to produce an adequate decrease in cGMP quickly enough to mediate the receptor potential (67). However, others have found that cGMP concentration decreases sufficiently quickly to mediate the receptor potential (68,69). The most recent estimate of the dark free cGMP concentration of ROS is 6 micromolar (3); 90% of all cGMP is bound, thus, albeit the total cGMP pool is large, the free concentration is relatively small. Light activated PDE would be able to modulate this smaller pool easily given its K_m for cGMP of 78 micromoles (48).

According to the cGMP model, cGMP is high in the dark when Na conductance is high. Similarly, cGMP is low in light when Na conductance is low. If this hypothesis were true, when cGMP is introduced into the ROS, a transient depolar-

ization might be expected. This is in fact what is observed when cGMP is iontophoresed or pressure injected into the ROS (70). Slow cGMP injections cause slow depolarizations (70). Moreover, the depolarization time was directly proportional to the amount of cGMP injected (71) and the depolarization effect is antagonized by light as one might expect since light activates PDE (71). Nonhydrolyzable analogues of cGMP, when introduced into the cell, slow the kinetics of the light response by their competitive inhibition of PDE (70). Also, the kinetics of cGMP hydrolysis is similar to that of the light response (72). Fast injections of cGMP into ROS followed by a light flash increases the light response latency, suggesting that the additional load of cGMP required hydrolysis to produce a receptor potential (72).

One hypothesis related to the Ca theory is the notion of proton induced release of intracellular Ca stores (4). The hydrolysis of cGMP by light activated PDE releases a proton. It was proposed that enough protons would be formed to cause local changes in pH near the discs causing conformational changes of disc proteins facilitating release of Ca. Ca would then block the Na dark current. This hypothesis would have integrated the two major competing theories.

It should be noted that the Ca and cGMP theories were not mutually exclusive. Indeed, intracellular Ca and cGMP concentration seem related. The relationship between cGMP and Ca is antiparallel, low Ca is associated with high cGMP and high Ca is associated with low cGMP. The molecular mechanisms for this relation is poorly defined though theories abound. Ca inhibits guanylate cyclase (73). Ca enhances PDE activation by light exposure (74). cGMP decreases Ca binding to the discs (75).

Fesenko et al. (2) have performed more definitive experiments addressing the question of the final internal transmitter of phototransduction. An increase in permeability is observed when cGMP is topically applied to the cytosol side of ROS membranes patch-clamped inside-out. When Ca is applied in the absence of cGMP, no perturbations of the transmembrane conductance is observed. In addition, Yau and Nakatani (3) have shown that the cGMP dependent cationic conductance is light suppressible. These results, of course, strongly imply that cGMP is the final mediator of phototransduction. Nonetheless, there are still unanswered questions. The role of Ca in phototransduction remains unclear. So, that cGMP affects the plasma membrane directly and that Ca appears to have no direct effects does not exclude Ca from playing an important role in phototrans-

duction. Equally unclear is the role of protons in photo-transduction in relation to these recent findings.

It is the purpose of these experiments to examine the response of the receptor potential to pressure injections of protons into the ROS of Bufo Marinus and to discuss some of the possible molecular mechanisms that may account for the observed effects.

II. MATERIALS AND METHODS

Wild Bufo Marinus are allowed to dark adapt in excess of two hours. The animals are decapitated and double pithed. Under an infra red light source and using an infra red image converter, the eye is enucleated and sectioned coronally. The retina is halved with one section containing the optic disc. The portion of retina without the optic disc is halved.

The patches of retina are mounted to Whatman No.1 filter paper receptor side up and placed in a bathing solution of amphibian Ringer's solution (NaHCO_3 25 mM, Na_2HPO_4 2.3 mM, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.8 mM, glucose 10 mM, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 mM, Na_2SO_4 0.6 mM, NaCl 78 mM, KCl 2.5 mM, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 1.0 mM) bubbled with 95% O_2 /5% CO_2 and adjusted to pH 7.1 with NaOH.

The intracellular recordings and injections of protons and control solutions are made with single barrel glass pipet microelectrodes. The microelectrodes are fashioned using a Takahashi Seiki Kogyo Co. Microelectrode Puller Model EH-12. The heating intensity and time are determined empirically. The microelectrodes are then filled with either the control solution, 25 mM KCl adjusted to pH 7.1 with NaOH, or proton solution, 25mM KCl adjusted to pH 2.0 with HCl. The imped-

ance of the microelectrodes to direct current is typically on the order of 100 megaohms.

The retina preparation is placed in a bathing chamber. The microelectrode is mounted to a Leitz micromanipulator. Both the bathing chamber and micromanipulator are on a screened table that has been placed on a pneumatic suspension system. The microelectrode is lowered onto the preparation and either spontaneously enters the ROS after contact with the ros surface or is oscillated into the cell using negative capacitance feedback applied as close to the contact point as possible to ensure entry into the outer segments. Responses from ros are obtained when the electrode was within 20 micrometers of the retinal surface.

The signal is processed through a WP Instruments Model M701 Microprobe System. The signal is visualized on a Tektronix D-10 Dual Beam Oscilloscope. The data are recorded onto a Hewlett-Packard 3960 FM Tape Recorder and tracings are made on a Hewlett-Packard 7402A Pen Writer. The whole retina preparation is stimulated with flashes of white light, 0 log units = 80 microw per square centimeteres or two million effective photons per sec per rod. Typically the light stimulus is of intensity -4 log units and lasts one hundred milliseconds or 20 effective protons per rod. Light

intensity is manually manipulated by a servo- controlled system. A Tektronix Waveform Generator is used to control the duration and the timing of the light flash through an electromagnetic shutter and pressure pulse through a solenoid valve. The pressure source is a nitrogen tank on which the pressure may be adjusted manually.

It is empirically determined that a pressure pulse of 1.2 sec at 100 PSI is adequate to result in injection of test solutions into ROS. For test solution of pH 2.0, it is calculated that each injection introduces millions of protons using previous estimates of injection volume (76). Calculation is shown in appendix.

III. RESULTS

A. The Effects of Proton Injection on Resting Potential

The test solution, 25 mM KCl adjusted to pH 2.0, is injected by a train of 21 pressure pulses. Each pulse is 100 PSI and lasts for 1.2 seconds. A typical response in the dark-adapted ROS is shown in Fig. 4. In all records, the top tracing is the transmembrane potential and the bottom tracing shows light flashes as upward deflections and pressure pulses as downward deflections. In four experiments, the ROS hyperpolarizes in response to the injections of protons. A very small hyperpolarization is apparent with the first few pulses and, with successive injections, the negative deflection from the baseline resting potential becomes more prominent. Maximal hyperpolarization, reached shortly after the final injection, is approximately 5 mV. Eventually, the resting potential returns to the original baseline 21.2 sec after the final injection.

Fig. 5 shows time scale enlargement of Fig. 4. The hyperpolarization response is composed of two components. 1) There is rapid hyperpolarization response of 3 mV in the first 500-750 msec of injection. 2) The response plateaus

and then a small depolarization component measuring 1 mV is seen in the final 250-450 msec of injection. The response of the membrane potential is seen immediately after the injection artifact (upward deflection); latency time is immeasurable. Though the magnitude of the perturbations in membrane potential is on the same order of magnitude as the background noise, the temporal relationship between the above described responses and injection implies that the hyperpolarizations are in response to proton injection.

Tracings of pressure injections of 25 mM KCl adjusted to pH 7.1 by a train of 20 pressure pulses at 100 PSI for 1.2 seconds is shown in Fig. 6. In three experiments, injection of solution of pH 7.1 results in no measurable perturbation in membrane potential. There is an insignificant, non-specific increase in noise level during the injection period, but the resting potential is otherwise undisturbed.

B. The Effects of Proton Injection on the Light Response

Fig. 7 shows records from injection of 25 mM KCl of pH 2.0 by a train of 13 pressure pulses at 100 PSI of 1.2 second duration immediately prior to light flash of intensity -4 log units for 100 msec. In five experiments, the response to proton injection prior to the light flash is similar to that in the dark adapted ROS in Fig. 4 and 5. The resting

potential is increasingly hyperpolarized with injections. Within an injection period, the membrane potential undergoes the same two component response described above. Initially, a 3 mV hyperpolarization in 500-750 msec followed by a slight depolarization 2 mV in 250-450 msec. (Fig. 8)

The first light flash response is a 15 mV hyperpolarization. Recovery is complete within 8.0 sec. The second light flash, 26.2 sec after the initial light flash, results in a response of amplitude 17 mV. The time for recovery is 7.5 sec. The third light flash, 49.3 sec after the initial light flash, results in a response of amplitude 17 mV. The time required for recovery is 5.8 sec.

The negative deviation from the baseline resting potential caused by protons is gradually attenuated and the resting membrane potential eventually recovers. Approximately 34.3 sec after the final injection, the resting potential returns to its original baseline.

Tracings from injections of solutions at pH 7.1 by a train of 22 pressure pulses, 100 PSI for 1.2 seconds, are shown in Fig 9. In six experiments, there is no change in baseline resting potential during injection or prior to light flash. The light response measures 19 mV. The recovery time is 7.1

sec. Light flashes prior to injections resulted in a 19 mV response with a recovery time of 7.1 sec.

C. The Effects of Proton Injection During Recovery

Records of response to injections of 25 mM KCl adjusted to pH 2.0 at 100 PSI for 1.2 seconds are shown in Fig 10. In six experiments, three injections are made during the recovery from light response to light stimulus of intensity -4 log units for 100 msec. The first injection is near the time of maximal hyperpolarization and results in a very small to nearly imperceptible change in recovery. The second injection is midway through the recovery process. Enlargement of this injection is shown in Fig. 11. The slope of recovery during the second injection is flattened compared to the same recovery segment following the second and third light flashes. The slope of recovery during the second proton injection is 3.3 mV/sec compared to 4.6 mV/sec and 5.0 mV/sec at corresponding times after light flash in the second and third responses respectively. The third injection is made when the recovery process is nearly complete. The configuration of the perturbation in potential Fig. 11 is comparable to that seen in the response of the dark adapted RGS to proton injection Fig. 5. Negative

deflection in response to the third injection is approximately 3 mV.

Note that the baseline potential to which the ROS returns after injections during recovery is nearly 3 mV more negative than that prior to injection. The resting potential eventually recovers 6.4 sec following the final injection.

In six experiments, injections of solution of pH 7.1 at 100 PSI for 1.2 seconds during the recovery process is seen in Fig. 12. There are no significant perturbations during injection. The slope of the recovery curve is unchanged from that prior to injection. The resting membrane potential is unchanged by injection.

IV. DISCUSSION

In the past two years, evidence has been presented that has proven cGMP, not Ca, is the internal messenger of phototransduction in vertebrate rod cells. Though some details of the nature of the interaction of cGMP with the light sensitive channels remains uncertain, cGMP is now firmly established as the internal transmitter.

The exact function of Ca in rod cells remains to be determined; some recent findings concerning the role of Ca in phototransduction challenge or are inconsistent with earlier findings. Ca may be involved with light adaptation since Ca modulates ROS enzyme activity ; it inhibits guanylate cyclase and enhances PDE light activation. cGMP and Ca seem to be inversely related; this, in part, may be due to Ca modulating ROS enzymes responsible for control of the intracellular cGMP concentration. Ca may function as a negative feedback signal. When the dark current is high, Ca flux through the cationic channels is high. Ca accentuates PDE light activation and inhibits cyclase activity. These two effects together act to reduce intracellular cGMP levels; therefore, fewer cationic channels are in the open state; the dark current decreases and Ca entry decreases.

The notion that protons may play a role in the phototransduction process is an old one. Radding and Wald in 1956 (77) and Matthews et al. in 1963 (78) found that rhodopsin, when photolyzed, take up protons. This effect, however, has been estimated to be very small by Ward and Ostroy (79) and McConnell et al. (80). Proton uptake ranges from one proton per photolyzed rhodopsin to one proton per 1000 photolyzed rhodopsins. It seems that the proton uptake effect, while detectable, is not significant. The proton uptake effect may be due to the protonation of basic amino acids which become exposed when rhodopsin is illuminated. The epsilon amino group of the lysine forming the Schiff-base may function in such a manner (81). Kinetic and equilibrium studies suggest protons are taken up in the metarhodopsin I-metarhodopsin II transition (82).

Mueller and Pugh (4) have proposed that protons generated from the hydrolysis of cGMP may induce the release of Ca from intracellular stores. One photolyzed rhodopsin results in the hydrolysis of 400,000 cGMP's each second and therefore generates 400,000 protons per second; a sufficeint number of protons may be formed to cause local changes in pH to alter membranes proteins. Although this theory is no longer a viable one since the Ca hypothesis for phototransduction has been disproven, their results require

explanation and that cGMP hydrolysis may result in local changes in pH is still plausible.

Assuming that protons have a role in phototransduction as a positive mediator of the light response, proton injection should have several consequences. In the resting, dark-adapted, rod cell, proton injection may cause hyperpolarizations depending on whether protons enhance the light reaction or actually mimic the light response. The recovery from light response after proton injection should be slowed compared to controls. Similarly, proton injections during recovery from light response should slow the recovery process by its hyperpolarizing effect.

Previous investigations of the role of protons in the ROS have given inconsistent results. Pinto and Ostroy (83) using superfusion technique found that reduction in pH of the perfusion fluid causes depolarization and increases light response amplitude. Liebman et al. (84) using superfusion and suction electrode technique found that the dark current is suppressed by protons and that the recovery from light response is retarded by protons. MacLeish et al. (85), iontophoresed protons into the ROS and found protons cause no change in dark current. The results of proton pressure injection experiments presented here are consistent

with those of Leibman et al. (84) and correspond well to the predicted results described above.

Protons injected into the resting ROS cause small hyperpolarizations during injections. The hyperpolarizations are small at first, 1-2 mV; with successive injections the hyperpolarizations become larger and the resting baseline potential becomes more negative. The consistent hyperpolarization-depolarization responses within any single injection prior to light flash is likely to be the result of intracellular buffers counteracting the injected proton load. With successive injections, the hyperpolarization effect becomes larger as protons accumulate and exceed the buffering capacity of the ROS. In addition, the diffusion of protons in the cell may also act to accentuate this phenomenon. Protons may initially form small pools near the site of injection. The protons eventually diffuse into the cytoplasm and are buffered.

Note that the potential eventually returns to its original baseline when the protons pool is sufficiently diluted and the buffering capacity of the ROS compensates for the protons introduced. Injections of protons in the dark adapted ROS prior to light flash give the same result.

Proton injection causes a decrease in the amplitude of the light response, 15 mV vs 17 mV. The light response is initiated at a potential 2-3 mV more negative than controls, therefore, it seems that the smaller light response is due to the hyperpolarization induced by proton injection prior to light flash. The recovery phase of the light response after proton injection is prolonged, 8.0 sec vs 5.8 sec. Since the ROS is exposed to the light flash before the resting potential has returned to the original baseline, this effect can be attributed to protons. The resting potential does not recover until 34.3 sec later. When the resting potential recovers, the light response is comparable to the control responses in both amplitude and rate of recovery.

Perhaps most interesting are the results of proton injections during recovery from light response. Protons injected during maximal light response have little to no effect. Protons injected during partial recovery cause slowing of the rate of recovery during the injection period. Protons injected when the rod cell has nearly completely recovered results in hyperpolarizations similar to the effect seen in the resting dark adapted ROS.

At the peak amplitude of light response, the number of closed cationic channels is maximal. Protons injected at this point would have the least immediate effect since any contribution to hyperpolarization would be proportionally very small since nearly all the channels are closed prior to the introduction of protons. Also, at peak light response, the driving force toward the K equilibrium is minimized. If the ROS were clamped at the voltage of peak response, the effect of protons would be minimized.

As the rod cell begins to recover, more channels reopen, and the number of channels in the open state begins to approach that in the resting ROS. Protons introduced at this point are proportionally more significant; in addition, since the potential is more depolarized, the drive toward the K equilibrium becomes greater. Midway through recovery, protons cause a decrease in the normal rate of recovery as reflected by the slope of recovery.

As the ROS nears complete recovery from light response, the response to proton injections becomes similar to that seen in the resting ROS; the number of open channels at this point is comparable to that in the resting ROS, therefore, protons exert an effect of similar magnitude to that seen in the resting ROS. The rod cell is near maximal depolar-

ization and the driving force toward the K equilibrium is greatest. The resting potential becomes 3-4 mV more negative in response to proton injection prior to light flash. Following the light response, the resting potential to which the ROS returns remains slightly more negative than the original baseline. This proton effect is eventually diminished and the ROS returns to the baseline potential 8.1 sec after the final injection.

In summary, the observed perturbations in membrane potential and kinetics of light response recovery in all three types of experiments are consistent with those predicted by the hypothesis that protons may be involved as a positive mediator of phototransduction.

There are several possible sites of action of protons in the ROS. Protons could directly interact with the cation channels. This would suggest that protons would in some manner alter the channel such that conductance is decreased. Direct action is consistent with the lack of latency period when protons are injected. Assuming that this is the mechanism of action, the distinction between experimental artifact and physiology must be made. It can be argued that this is a physiologic process. Each injection introduces millions of protons into the ROS. Since one photolyzed

rhodopsin induces release of 400,000 protons, the number of injected protons is in the physiologic range. Local perturbations in pH secondary to the generation of millions of protons from the hydrolysis of cGMP is plausible.

It may also be argued that this data is artifactual. Measuring pH by injection of pH sensitive dye into the ROS, Yoshikami and Hagin (85) find that there is no significant changes in pH after illumination. An explanation presented by the authors is that protons are consumed in a concomitant reaction, a transfer of a phosphate group. The technique used by Yoshikami and Hagin is, however, an indirect and artificial means to assess the role of protons in the ROS. Arguably, injection of protons is a more direct means to approach this problem. pH dependence of cationic channels by patch-clamp experiments may provide useful data for the determination of the function of protons in the ROS.

While it has been determined that cGMP maintains the cation channels in the open state, the manner in which channels are closed has not been clarified. It is possible that the hydrolysis of cGMP may induce dissociation of the cyclic nucleotide from the channel causing closure in a push-pull manner. However, protons, in conjunction with the dissociation of cyclic nucleotides from the channel, may

induce or enhance channel closure. pH dependence of cation closure was not tested by Fesenko et al (2).

The proton load could effect the equilibrium of cGMP hydrolysis. If protons had a significant effect on this level, it would inhibit the hydrolysis of cGMP by LeChatelier's principle. This would increase cGMP concentration and therefore increase cationic conductance resulting in a tendency toward the Na equilibrium potential. This is inconsistent with the results.

The relation between protons and rhodopsin has been noted. This is an unlikely site for protons to action for several reasons. First, the reaction described states that a rhodopsin molecule, in the presence of light, takes up a proton. If protons were to effect the equilibrium, they would promote the formation of photolyzed rhodopsin. This necessarily implies that the light activated cascade is the pathway of transmission of the signal. A latency period should then be observed; this is not supported by the data. Second, this mechanism requires light exposure for protons to have effect and cannot account for the effect of protons on the resting dark adapted ROS. Third, if protons act on rhodopsin, then a larger hyperpolarization response might be

expected because of the tremendous amplification of the signal from photolyzed rhodopsin and cGMP.

The lack of latency period and relatively small responses to protons argue against protons having significant effect at the transducin or PDE level.

An interesting possibility is the notion that protons may have a blocking effect on the Na/Ca exchange mechanism as mentioned by Mueller and Pugh. If this were the case, intracellular Ca concentration increases during proton injection since Ca extrusion would be inhibited. The rise in intracellular Ca would enhance light activated PDE activity and more cGMP would be hydrolyzed. This mechanism would account for the retarded recovery phase of light response and the inhibition of recovery when protons are injected during recovery. This mechanism is also consistent with the tendency of the membrane potential to return to baseline potential within a single injection. If Ca acts as a negative feedback signal and protons block the extrusion of Ca, the enhanced PDE activity reduces cGMP, the channels close, and Ca entry into the cell is diminished. Thus, cation permeability is restored and the rod depolarizes to return to near the baseline potential. There are, however, several inconsistencies in this scheme. This mechanism acts

through PDE and therefore some latency would be expected and none is observed. The rod cell hyperpolarizes in darkness in response to protons and, if Ca were involved, it would act through PDE by enhancing light activated PDE activity; the data suggest that light is not required for protons to exert an effect.

Similarly, if protons were to effect the proposed proton-Ca exchanger (4) they would act through Ca and necessarily through PDE. Since there is no latency period, this mechanism is unlikely to account for these data.

In conclusion, the results of proton injection experiments suggest that protons may be involved in the phototransduction process. The kinetics of the proton effect imply that protons act directly on the cationic channels. The mechanism by which protons act is uncertain. The results presented here suggest that protons, in conjunction with cyclic nucleotide dissociation from channels, may induce or enhance the closure of the cationic channels of the vertebrate ROS. Whether this effect is physiologic or artifactual will depend on the actual pH change at the channel resulting from the action of light which is so far unknown. Although it has been suggested that the ROS buffering capacity is sufficient to prevent significant

fluctuations in pH, true measurement under physiologic conditions has yet to be made.

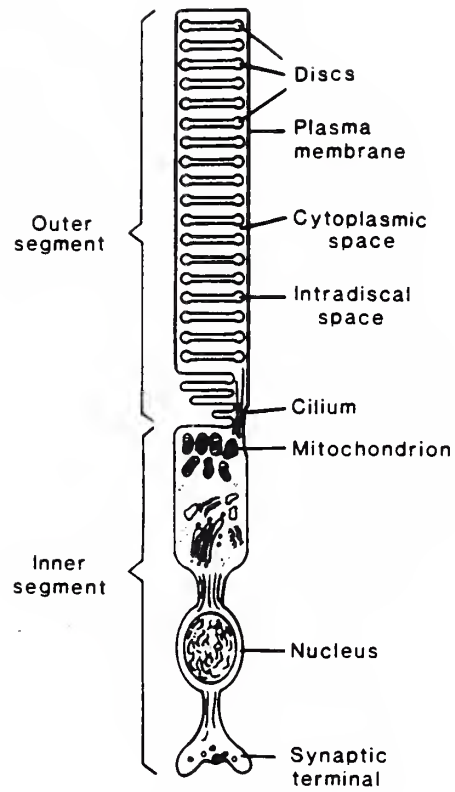


Figure 1

Figure 1. Schematic of a vertebrate rod cell. The cell consists of a synaptic terminal for transmission of signals to secondary neurons of the retina, inner segment containing the metabolic machinery, and the outer segment containing the discs on which rhodopsin is located. The inner and outer segments are connected by a ciliary stalk. (86)

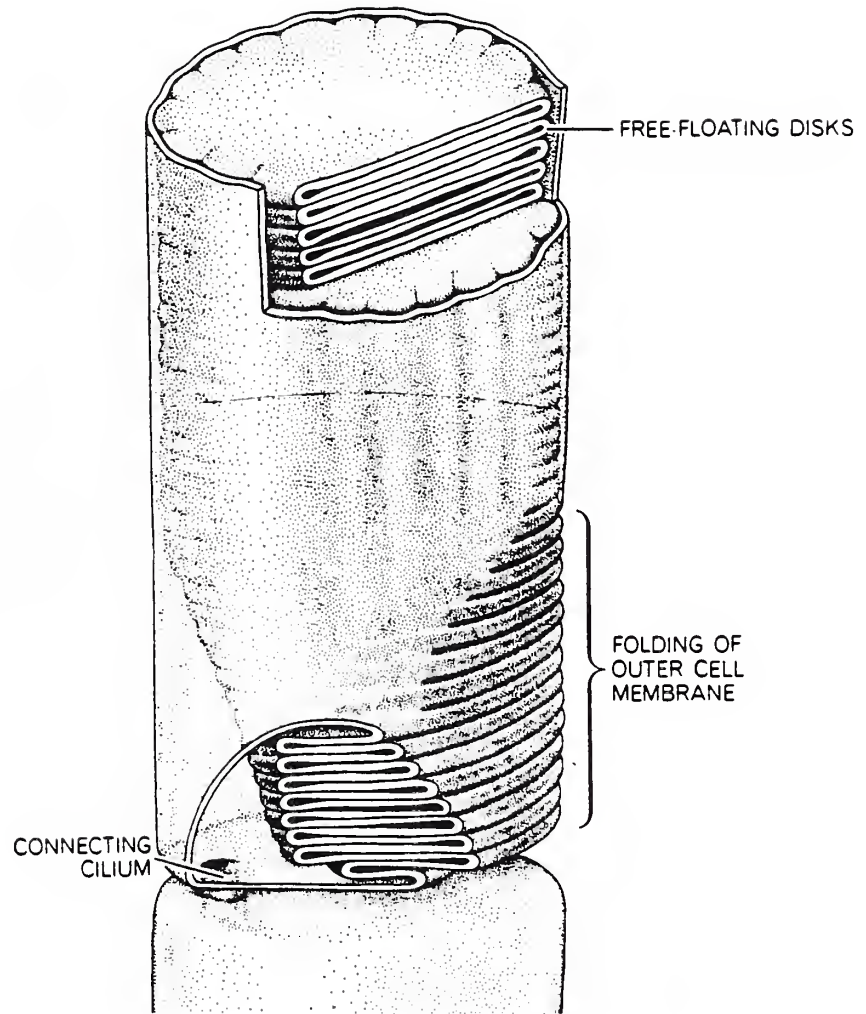


Figure 2

Figure 2. The outer segment of a rod cell. Discs are formed by infoldings of plasma membrane. The folds pinch off so that discs become free floating. (87)

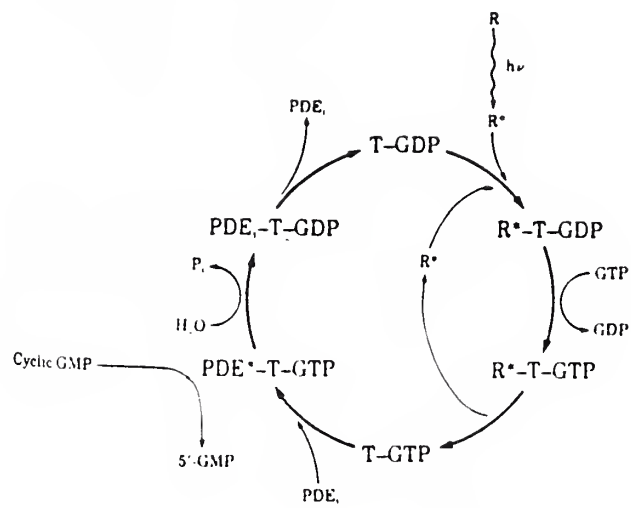


Figure 3

Figure 3. The light amplification cycle. Starting at 1 o'clock, rhodopsin (R) is activated by light ($h\nu$). Photolyzed rhodopsin (R^*) directly interacts with transducin-GDP (T-GDP) and induces the exchange of GDP for GTP which transforms transducin to the activated form, transducin-GTP. Photolyzed rhodopsin is then free to activate more transducin-GDP complexes. Transducin-GTP activates phosphodiesterase (PDE) which hydrolyzes cGMP to GMP. The GTPase activity of transducin hydrolyzes GTP to GDP and PDE dissociates from the complex and transducin-GDP re-enters the cycle. (41)

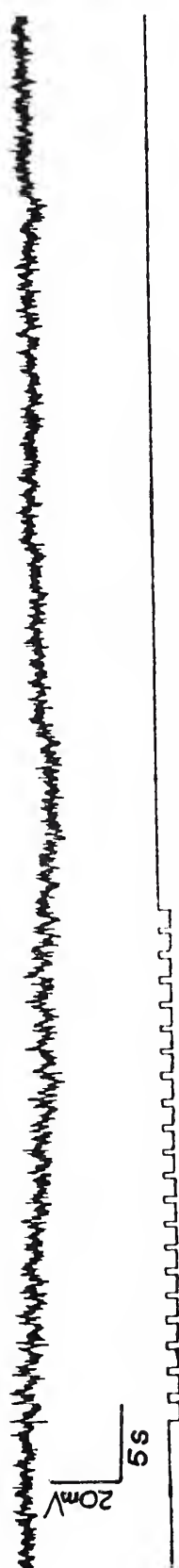


Figure 4

Figure 4. Injection of solution of pH 2.0 into the resting dark adapted ROS. In all records, light flashes are shown as upward deflections in the lower tracing, pressure pulses are shown as downward deflections in the lower tracing. The membrane potential becomes more negative over the time course of injections. Each injection causes small hyperpolarizations. The potential eventually recovers with time.

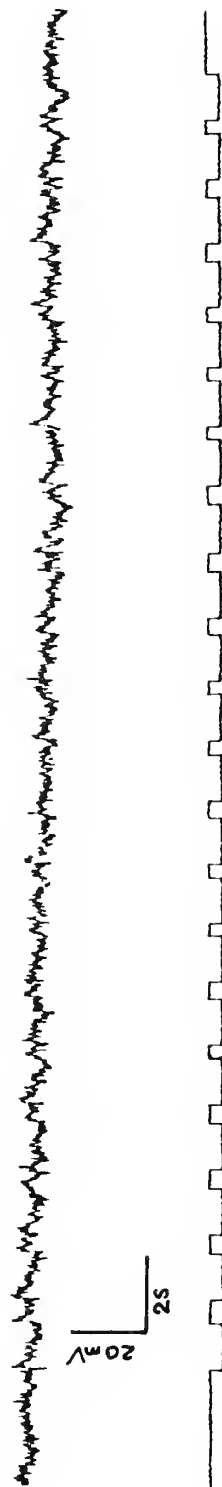


Figure 5

Figure 5. Time scale enlargement of Fig. 4. Note the temporal relation of the hyperpolarizations and injections. Hyperpolarization responses may be seen in any single injection period. Membrane potential becomes progressively more negative with successive injections.

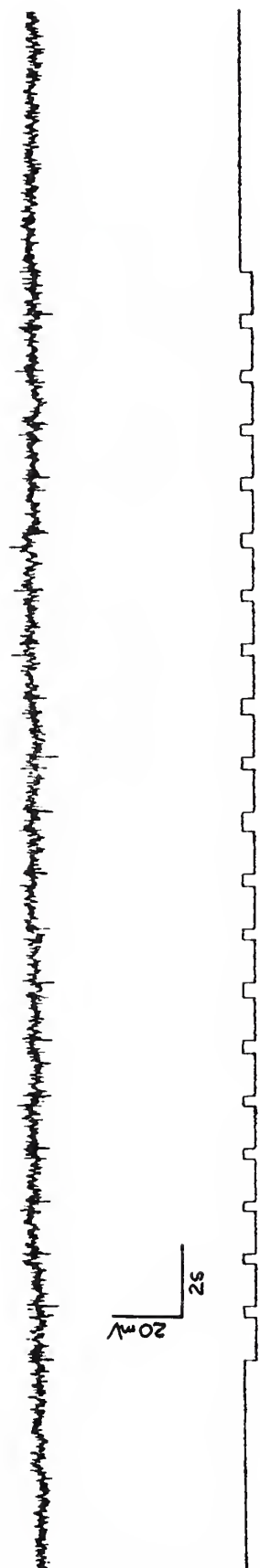


Figure 6

Figure 6. Injection of solution of pH 7.1 into the resting dark adapted ROS. Noise level is increased during the injection periods but the membrane potential is otherwise undisturbed.

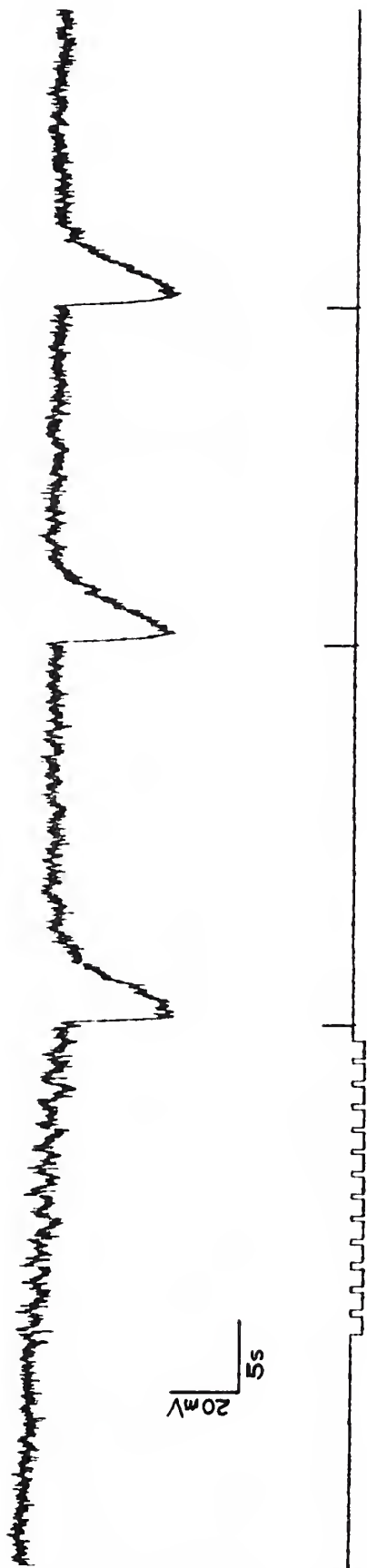


Figure 7

Figure 7. Injections solution of pH 2.0 into the dark adapted ROS prior to light exposure. The membrane potential hyperpolarizes in a similar manner to that seen in the resting dark adapted ROS (Fig. 4, Fig 5). The amplitude of the light response is decreased and the recovery time increased. The second and third light flashes show that both the amplitude of the light response and recovery time returns to baseline levels after a period of time.

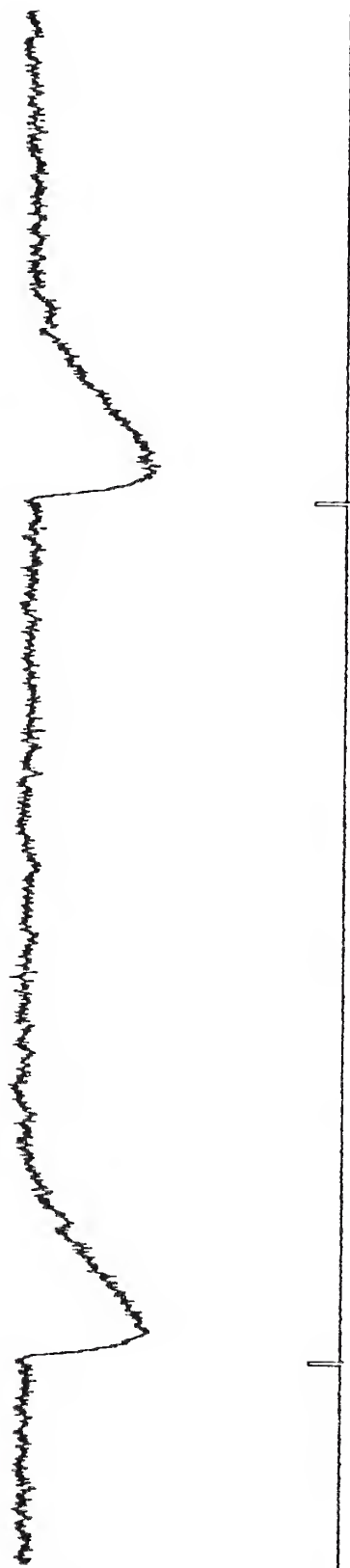
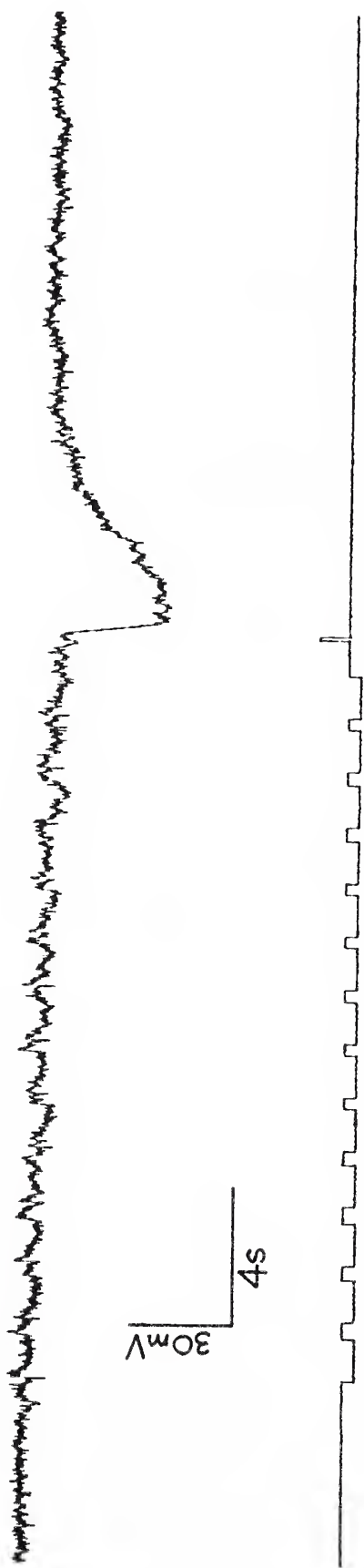


Figure 8

Figure 8. Time and voltage scale enlargement of Fig. 7. Upper record is of membrane response to proton injection. Lower record is of two subsequent responses to light.

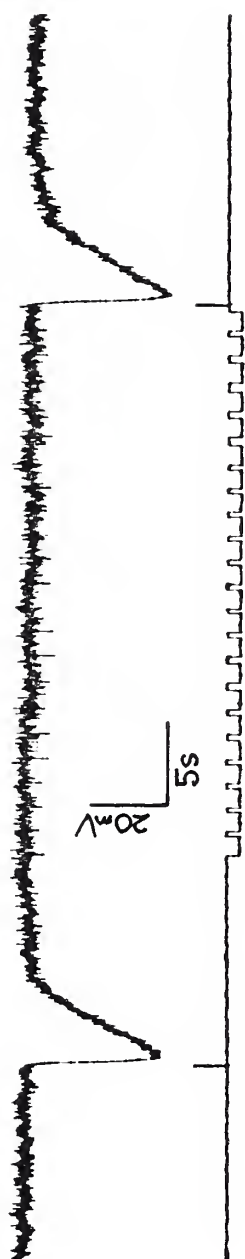


Figure 9

Figure 9. Injection of solution of pH 7.1 prior to light exposure. Injections results in no perturbation in membrane potential from resting baseline values.



Figure 10

Figure 10. Injection of solution of pH 2.0 during recovery from light response. At maximal hyperpolarization, there is minimal response to proton injection. As the potential recovers, response to proton injection becomes more prominent. When the potential has returned to near baseline values, the membrane response to proton injection is comparable to that seen when protons are injected in the resting dark adapted ROS (Fig. 4, Fig 5).

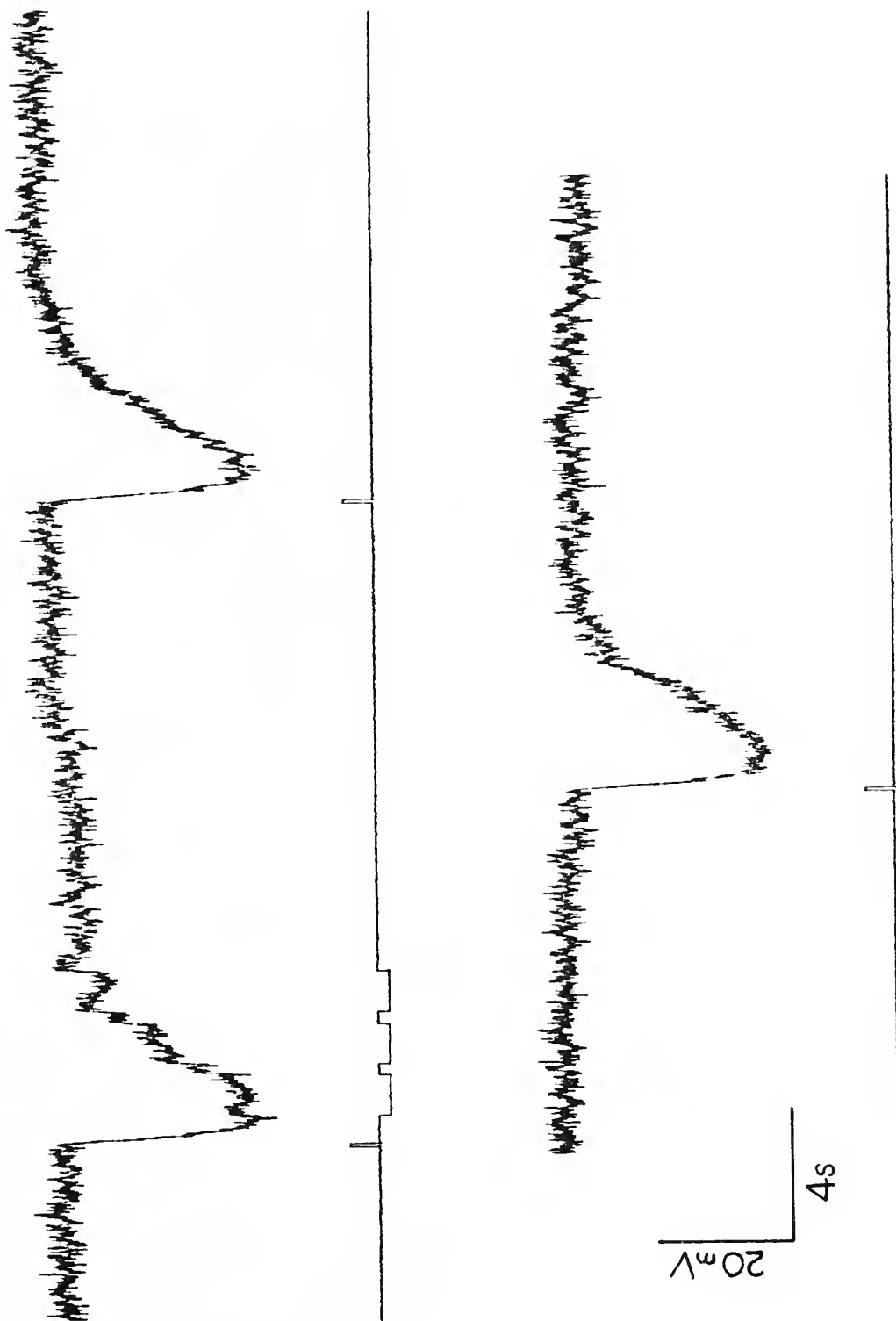


Figure 11

Figure 11. Time and voltage scale enlargement of Fig. 9. Note the slope of the recovery from light response is decreased during the second proton injection compared to the slopes in the same time period in subsequent light flashes.

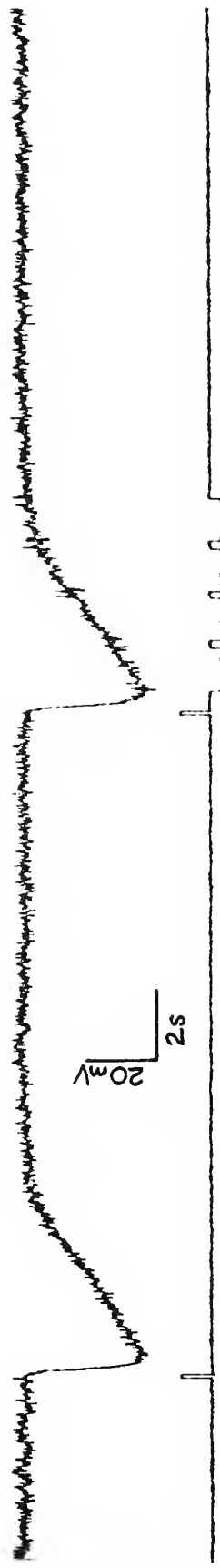


Figure 12

Figure 12. Injection of solution of pH 7.1 during recovery from light response. Recovery is unaffected by injections.

APPENDIX

From Shimoda et al. (76),

400,000 Pascals (=59 PSI) pulse of 200 msec duration injects
300 molecules of 2.5 micromolar PDE.

Therefore,

$$\frac{300}{(2.5 \times 10^{-6} \text{ moles/l})(6.023 \times 10^{23} \text{ no./mole})}$$
$$= 2.0 \times 10^{-16} \text{ l injected.}$$

Assuming that

1. pressure of injection and volume injected are linearly related
2. duration of pressure pulse and volume injected are linearly related
3. there is no covariance of pressure of injection and duration of injection,

for 100 PSI and 1.2 sec injections,

$$\frac{100}{59} \times \frac{1.2}{0.2} (2 \times 10^{-16} \text{ l}) = 3 \times 10^{-15} \text{ l/injection}$$

For each injection of 100 PSI, 1.2 sec duration of solution of
pH 2,

$$(3 \times 10^{-15} \text{ l})(10^{-2} \text{ M/l})(6.023 \times 10^{23} \text{ no./mole})$$
$$= 18 \times 10^6 \text{ protons/injection.}$$

This calculation also assumes that the viscosity of the PDE solution used in the injection estimates of Shimoda et al. is comparable to that of the test solutions in these experiments.

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